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Original Contribution

The use of ebselen for radioprotection in cultured cells and mice

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ABSTRACT

Ionizing radiation induces the production of reactive oxygen species (ROS), which play an important causative role in cell death. Therefore, compounds that control the level of ROS may confer radioprotective effects. Ebselen, a seleno-organic compound, has been shown to protect against cell injury caused by ROS. The objective of this study was to examine the effects of ebselen on radiation-dependent toxicity. We investigated the protective role of ebselen against ionizing radiation in U937 cells and mice. Upon exposure to 20 Gy of γ-irradiation, there was a distinct difference between untreated cells and the cells pretreated with 5 μM ebselen for 2 h with respect to viability, cellular redox status, and oxidative damage to cells. When cells were exposed to 2 Gy of γ-irradiation, there was a distinct difference between the untreated cells and the cells pretreated with ebselen with respect to apoptotic features and mitochondrial function. Ebselen administration for 14 days at a daily dosage of 10 mg/kg provided substantial protection against killing and oxidative damage to mice exposed to whole-body irradiation. These data indicate that ebselen may have great potential as a new class of in vivo, non-sulfur-containing radiation protector.

Ionizing radiation has been shown to generate reactive oxygen species (ROS) in a variety of cells [1]. When water, the most abundant intracellular material, is exposed to ionizing radiation, decomposition reactions occur and a variety of ROS, including superoxide, hydroxyl radicals, singlet oxygen, and hydrogen peroxide, are generated [2]. The secondary radicals formed by the interaction of hydroxyl radicals with organic molecules may also be of importance [1,2]. These ROS have the potential to damage critical cellular components such as DNA, proteins, and lipids, which eventually results in physical and chemical damage to tissues that may lead to cell death by necrosis and apoptosis or to neoplastic transformation [3,4].

Because ROS seem to be the mediators of the cellular damage induced by ionizing radiation, compounds that regulate the fate of such species may be of great importance in the protection of cells against radiation-induced damage. The search for agents that protect against ionizing radiation is important to those at risk by virtue from environmental exposure or health-related treatments and for the scientific study of the mechanism of radiation injury and cytotoxicity [5]. Although no radioprotective drug available today has all the requisite qualities of an ideal radioprotector, sulfhydryl radioprotectors such as cysteine, *N*-acetylcysteine, cysteamine, cystamine, aminoethylisothiourea dihydrobromide, and mercaptoethyl guanidine are the best radioprotectors known today. However, their use encounters two great difficulties: their toxicity and the short period during which they are active [6].

The organoselenium compound ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) has antioxidant properties [7]. This compound has been extensively studied as a mimic of glutathione peroxidase (GPx) and thioredoxin peroxidase, and it is known to exhibit substantial pharmacological effects [8,9]. Unlike inorganic selenium and selenomethionine, ebselen is not toxic to mammals, most likely because it contains bound selenium [10]. In this study, the role of ebselen in cellular and in vivo defense against ionizing radiation was investigated using U937 cells and mice, respectively. To determine differences in sensitivity to the toxic effects of ionizing radiation between U937 cells treated with ebselen and untreated control cells, viability, cellular redox status, oxidative damage to cells, modulation of apoptotic features, and mitochondrial damage were examined upon exposure of the cells to ionizing radiation. Ebselen was also administered to mice before treatment with ionizing radiation, and the in vivo radioprotective effect was assessed. Our results demonstrate that ebselen may play an important role in regulating the cell death induced by ionizing radiation and may have great potential as a new class of in vivo, non-sulfur-containing radiation protector.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DHE, dihydroethidium; DHR 123, dihydrorhodamine 123; DMSO, dimethyl sulfoxide; DNPH, 2,4-dinitrophenylhydrazine; DPPP, diphenyl-1-pyrenylphosphine; FITC, fluorescein isothiocyanate; GPx, glutathione peroxidase; H and E, hematoxylin and eosin; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; JC-1, 5,5′,6,6-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide; MDA, malondialdehyde; MPT, mitochondrial membrane potential transition; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances; TRITC, tetramethylrhodamine isothiocyanate; Trx, thioredoxin.

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Materials and methods

Materials

Ebselen, 2,4-dinitophenylhydrazine (DNPH), propidium iodide (PI), xylenol orange, avidin-conjugated tetramethylrhodamine isothiocyanate (TRITC), 4′,6-diamidino-2-phenylindole (DAPI), anti-rabbit IgG TRITC-conjugated secondary antibody, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dihydroethidium (DHE), diphenyl-1-pyrenylphosphine (DPPP), dihydrorhodamine (DHR) 123, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1), and Alexa Fluor 488 annexin V/PI were purchased from Molecular Probes (Eugene, OR, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling (Beverly, MA, USA).

Cell culture and cytotoxicity assay

Human premonocytic U937 cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, penicillin (50 units/ml), and 50 μg/ml streptomycin at 37°C in a 5% CO_2/95% air humidified incubator. For the cytotoxicity assay, U937 cells were first grown on a 96-well plate at a density of 2×10^5 cells/well. After optimization of culture conditions for confluence, ebselen was applied to the cells and cells were incubated for an additional 2 h at 37°C. After incubation, cells were irradiated at room temperature with a ^{137}Cs source at a dose rate of 1 Gy/min. After 48 h of irradiation, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS)/phenazine methosulfate solution was added and incubated for another 4 h at 37°C. The absorbance was read in an ELISA plate reader at 490 nm with a 620 nm reference.

Mice

Adult male ICR mice were housed five per cage in a climate-controlled, circadian rhythm-adjusted room and allowed food and water ad libitum. The animals were, on average, 50 to 70 days of age and weighed between 20 and 30 g at the time of irradiation. Experiments on mice were conducted according to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council, Washington, DC.

Toxicology of ebselen and whole-body irradiation

To determine its maximum tolerated dose, solutions of ebselen were freshly prepared in DMSO. Two groups of 15 mice each received either ebselen or DMSO. Ebselen was administered before irradiation at a dose of 10 mg/kg in 100 μl of DMSO once daily for 2 weeks. Control mice were given DMSO and all injections were administered orally by gavage using a sonde. Survival was assessed up to 20 days after injection without irradiation. To determine survival after whole-body irradiation, the same protocol for the ebselen administration was applied and then the groups of 15 mice were transferred to round Plexiglas containers (30.5 cm in diameter and 10.5 cm in height) with holes for ventilation. After irradiation with a ^{137}Cs source at a dose rate of 1 Gy/min, the mice were returned to climate-controlled cages for observation. Survival was assessed 20 days after irradiation. For the hematoxylin and eosin (H and E) stain and determination of oxidative damage mice were sacrificed 5 days after γ-irradiation.

Cellular redox status

Superoxide anion production was measured using the fluorescent probe DHE with a fluorescence microscope. Cells in PBS were incubated for 30 min at 37°C with 5 μM DHE. For in vivo visualization of ROS generation in tissues, tissues were perfused with 10 μM DHE for 25 min. The DHE fluorescence from cryosections of tissues was observed with a fluorescence microscope. Intracellular peroxide concentrations were determined using the ferric-sensitive dye xylenol orange, as previously described [11].

Cellular oxidative damage

The protein carbonyl content was determined spectrophotometrically using the DNPH labeling procedure as previously described [12]. The presence of thiobarbituric acid-reactive substances (TBARS) was determined as an indicative marker of lipid peroxidation. Samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBARS [13]. Lipid peroxidation was also estimated using the fluorescent probe DPPP as described by Okimoto et al. [14]. After U937 cells (1×10^6 cells/ml) were incubated with 5 μM DPPP for 15 min in the dark, the cells were exposed to ionizing radiation. A Zeiss Axiovert 45CFL inverted microscope was used to capture images of DPPP fluorescence by reactive species in the fluorescence DAPI region (excitation, 351 nm; emission, 380 nm). The levels of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in U937 cells were estimated using a fluorescence binding assay as described by Struthers et al. [15]. After exposure of U937 cells to ionizing radiation, cells were fixed and permeabilized with ice-cold methanol for 15 min. DNA damage was visualized with avidin-conjugated TRITC (1:200 dilution) using a fluorescence microscope at 540 nm excitation and 588 nm emission.

Apoptosis assay

DAPI staining was used for apoptotic nuclei determination. U937 cells were collected at 2000 g for 5 min, washed once with cold PBS, fixed in ice-cold methanol/acetic acid (1/1, v/v) for 5 min, and stained with 0.8 μg/ml DAPI in the dark. Morphological changes in apoptotic cells were analyzed using a Zeiss Axiovert 45CFL microscope in the fluorescence DAPI region (excitation, 351 nm; emission, 380 nm). Alexa Fluor 488 annexin V/PI was used to measure the relative distribution of apoptotic and necrotic cells. The cell suspension was double-stained with annexin V-fluorescein isothiocyanate (FITC) and PI and analyzed by flow cytometry. To determine the degradation of chromosomal DNA into nucleosome-sized fragments, a 500-μl aliquot of the lysis buffer (100 mM Tris-HCl, pH 8.5, EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K) was added to the cell pellet (2×10^5 cells) and incubated at 37°C overnight. DNA was obtained by ethanol precipitation, separated on a 0.8% agarose gel, and visualized under UV light. DNA fragmentation was also determined using the diphenylamine assay as previously described [16].

Immunoblot analysis

Proteins were separated on a 10 to 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently subjected to immunoblot analysis using appropriate antibodies. The immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Mitochondrial damage

Mitochondrial membrane potential transition (MPT) was assessed using the mitochondrial-specific fluorescent probe JC-1 based on the method of Reers et al. [17]. To evaluate the levels of mitochondrial ROS U937 cells in PBS were incubated for 20 min at 37°C with 5 μM DHR 123. Cells were washed, resuspended in complete growth medium, and subjected to ionizing radiation. The cells were then incubated for